Bacterial and Human Cell Mutagenicity Study of Some $C_{18}H_{10}$ Cyclopenta-Fused Polycyclic Aromatic Hydrocarbons Associated with Fossil Fuels Combustion

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A number of isomeric $C_{18}H_{10}$ polycyclic aromatic hydrocarbons (PAHs), thought to be primarily cyclopenta-fused PAHs, are produced during the combustion and pyrolysis of fossil fuels. To determine the importance of their contributions to the total mutagenic activity of combustion and pyrolysis samples in which they are found, we characterized reference quantities of four $C_{18}H_{10}$ CP-PAHs: benzo[*ghi*]fluoranthene (BF), cyclopenta[*cd*]pyrene (CPP), cyclopent[*hi*]acephenanthrylene (CPAP), and cyclopent[*hi*]aceanthrylene (CPAA). Synthesis of CPAA and CPAP is described. The availability of reference samples of these isomers also proved to be an essential aid in the identification of the $C_{18}H_{10}$ species often found in combustion and pyrolysis samples. Chemical analysis of selected combustion and pyrolysis samples showed that CPP was generally the most abundant $C_{18}H_{10}$ isomer, followed by CPAP and BF. CPAA was detected only in pyrolysis products from pure PAHs. We tested the four $C_{18}H_{10}$ PAHs for mutagenicity in a forward mutation assay using *S. typhimurium.* CPP, BF, and CPAA were roughly twice as mutagenic as benzo[*a*]pyrene (BaP), whereas CPAP was only slightly active. These PAHs were also tested for mutagenic activity in human cells. In this assay, CPP and CPAA were strongly mutagenic but less active than BaP, whereas CPAP and BF were inactive at the dose levels tested. Also, the bacterial and human cell mutagenicity of CPAA and CPAP were compared with the mutagenicity of their monocyclopenta-fused analogs, aceanthrylene and acephenanthrylene. Although the mutagenicities of CPAP and acephenanthrylene are similar, the mutagenic activity of CPAA is an order of magnitude greater than that of aceanthrylene. **Key words:** diode-array spectrophotometric detection, forward mutation assay, fossil fuels combustion, human cell mutagenicity, polycyclic aromatic hydrocarbons. *Environ Health Perspect* 101:146–153(1993)

Cyclopenta-fused polycyclic aromatic hydrocarbons (CP-PAHs) are an important class of PAH that is abundant in the effluent from a number of different combustion systems and is also present in airborne particulate matter (*1–14*). A growing body of data has revealed that many CP-PAHs are mutagens and animal carcinogens and thus potentially pose a significant health threat to humans (*2,9–22*).

Using a combination of analytical techniques, we have identified several $C_{18}H_{10}$ CP-PAHs in products from different combustors and pyrolyzers operated over a wide range of conditions *(4–6).* Of the several $C_{18}H_{10}$ isomers possibly present, cyclopenta[*cd*]pyrene (CPP) was found in an earlier study to be the most important bacterial mutagen emitted from a jet-stirred/plug-flow reactor (*5*). In addition to CPP, one of the other $C_{18}H_{10}$ PAHs found in the sample was postulated to be benzo[*ghi*]fluoranthene (BF); however, the lack of reference standards prevented unequivocal identification.

To identify other $C_{18}H_{10}$ isomers and to determine their contribution to the overall mutagenicity of combustion and pyrolysis samples, we characterized reference samples of four $C_{18}H_{10}$ PAHs cyclopent[*hi*]acephenanthrylene (CPAP), CPP, cyclopent[*hi*]aceanthrylene (CPAA), and BF. In addition, we compared the bacterial and human cell mutagenicity of CPAA and CPAP with those of their monocyclopenta-fused analogs, aceanthrylene (AA) and acephenanthrylene (AP), to determine the effect of adding an additional fused cyclo-penta group. All PAHs were produced synthetically, except for BF, which was obtained commercially. Structures and nomenclature are shown in Figure 1.

We identified $C_{18}H_{10}$ isomers in combustion samples by GC/MS and HPLC with diode-array spectrophotometric detection. Results are presented for fuel-rich ethylene combustion products from a jet-stirred/plug-flow reactor. The four $C_{18}H_{10}$ PAH isomers were tested for mutagenicity in a forward mutation assay based on *Salmonella typhimurium.* They were also tested for mutagenic activity in human cells.

Methods

The jet-stirred/plug-flow combustor was designed to provide well-defined combustion conditions, and its use is part of an ongoing program aimed at understanding and controlling the combustion chemistry responsible for mutagen formation. Fuel-rich combustion in a jet-stirred reactor (fuel equivalence ratio, 2.37, C/O, 0.791) provides baseline input to a close-coupled, plug-flow reactor where continuing molecular-weight growth reactions occur. Detailed descriptions of this combustor are available elsewhere (*23–25*).

In this study, the fuel was ethylene (fuel equivalence ratio, 2.37; residence time, 5.69 msec; reactor temperature, 1628°K). We obtained samples from the plug-flow region of the combustor using an aspirated probe connected to a high-yield combustion sampler consisting of two refrigerated traps containing a total of 4 l of dichloromethane. The first trap was maintained at -30°C and the second at -68°C. Samples were concentrated in a 1-l Kuderna-Danish evaporative concentrator to the dissolution limit and filtered through a 0.2-µm fluorocarbon filter to remove particulates.

The HPLC system used for chemical analysis consisted of a Hewlett-Packard Model 1090 ternary gradient pumping system with 190–600-nm diode-array detector. It used HPLC operating software running on a Model 7994 Analytical Work-station. Injection volumes ranging from 1 to 25 µl could be selected. The HPLC column was a Vydac 201TP C18 type optimized for PAH separations (Vydac, Inc., Hesperia, CA; diameter, 4.6 mm; length 250 mm). The column was packed with 5 µm material of 300 Å pore size.

The mobile phase consisted of acetonitrile and water. The solvent program consisted of a linear ramp from 40% acetonitrile/60% water to 100% acetonitrile in 80 min. The flow rate was 1.0 ml/ min. UV-visible spectra (200–600 nm) were obtained at 0.10-sec intervals. Total absorbance chromatograms shown in this work were recorded over the 236–500 nm wavelength interval. The use of wideband

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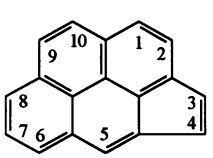
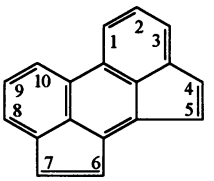
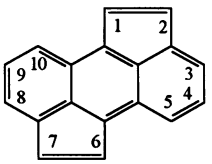


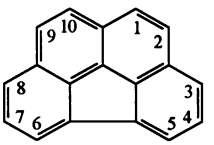
CPP
cyclopenta[*cd*]pyrene



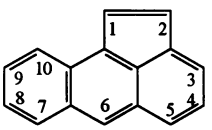
CPAP
cyclopent[*hi*]acephenanthrylene



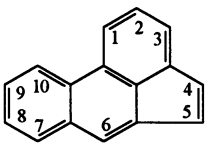
CPAA
cyclopent[*hi*]aceanthrylene



BghiF
benzo[*ghi*]fluoranthene



AA
aceanthrylene



AP
acephenanthrylene

Figure 1. Structure and nomenclature of $C_{16}H_{10}$ and $C_{18}H_{10}$ cyclopenta-fused polycyclic aromatic hydrocarbons studied.

diode-array detection for the analysis of polycyclic aromatic compounds has been reported elsewhere (*26*).

GC/MS consisted of an HP 5890 gas chromatograph connected to a model 5970 mass selective detector. Data acquisition and analysis were accomplished using a model 59970C MS ChemStation. The instrument was obtained from Hewlett-Packard (Palo Alto, CA). The GC column was a methyl-(5%-phenyl)silicone FSOT column (Quadrex, Inc.) with a length of 25 m, an i.d. of 0.25 mm, and a film thickness of 0.25 μm. Column temperature programming was from 40°C to 280°C at 10°C/min. Injector and transfer lines were at 280°C. Injection volume was 1.0 μl. HPLC solvents were Caledon HPLC grade obtained from American Bioanalytical (Natick, MA). BF was obtained from the Bureau of Community Reference (Brussels). AA, AP, CPP, CPAA, and CPAP were produced synthetically.

Mutagenicity Assays

The samples, dissolved in dichloromethane, were prepared for bioassay by adding a measured amount of dimethyl sulfoxide in a V-bottom vial. We evaporated the dichloromethane with a gentle stream of nitrogen until the total volume was reduced to that of the original dimethyl sulfoxide. Sample concentrations in dimethyl sulfoxide were typically 10–20 mg/ml.

We used the forward mutation assay to 8-azaguanine resistance in *S. typhimurium* strain TM677 to measure bacterial mutagenicity. Detailed protocols for measuring forward mutation have been described previously (*27–29*). Briefly, we suspended exponentially growing bacteria in medium in the presence of test sample for 2 hr. Samples at a number of concentrations ranging from 0.10 to 20 μg/ml, depending on their mutagenic potency, were exposed to the bacteria in the presence of 5% (v/v) Aroclor-induced post-mitochondrial supernatant (PMS). Cultures containing PMS had an NADPH-generating system. After 2 hr we resuspended cells in fresh medium. Aliquots were plated in the presence or absence of the selective agent (8-azaguanine, 50 μg/ml). We performed each assay in duplicate with triplicate plates for each experiment.

We counted colonies at 48 hr and determined the mutant fraction (MF) as the number of colonies formed under selective conditions divided by the number of colonies formed under nonselective conditions multiplied by the dilution factor. The assay was ruled positive if at any sample concentration: 1) the number of mutant colonies in the treated cultures was greater than the number of mutant colonies in the control cultures with greater than 99% confidence as calculated by Poisson distribution, and 2) the MF exceeded the 95% upper confidence limit for the cumulative historical control (14×10^{-5}).

We tested compounds for mutagenic activity at the thymidine kinase (*tk*) locus in MCL-3 cells. These cells were derived from an L3 variant (*30*) of AHH-1 $TK^{+/-}$ cells, a metabolically competent line of human B-lymphoblastoid cells containing cytochrome CYP1A1 (*31*). MCL-3 cells were derived from L3 cells by transfection of the plasmid pME23, which contains cDNAs for human cytochromes CYP1A2 and CYP2A6 and microsomal epoxide hydrolase (*32*). L3 cells have a 2-fold higher CYP1A1 activity and a lower background mutation frequency at the *tk* locus than AHH-1 cells.

We adapted the assay protocol from the procedure used with AHH-1 cells (*33*). Exponentially growing cells were incubated in duplicate cultures (6×10^6 cells per 12-ml culture) containing different concentrations of test compound for 28 hr. We terminated this treatment phase by centrifuging the cells and resuspending them in 50 ml fresh media. One day later we counted the cells and added sufficient fresh media to reduce the cell concentration to 2×10^5 cells/ml. The cells were grown for an additional 2 days without further dilution to allow for phenotypic expression of the mutants. Finally, we plated the cells from each experiment in duplicate 96-well microtiter plates in both the presence and absence of 4.0 g/ml trifluorothymidine as the selective agent to measure mutagenicity and plating efficiency, respectively. For the mutagenicity measurements 20,000 cells/well were plated; for measurements of plating efficiency, 2 cells/well were plated. We incubated the plates for 13 days and scored them for the presence of a colony in each well. The positive control was 5 g/ml benzo[*a*]pyrene, and the negative control was 60 μl of the solvent vehicle (dimethyl sulfoxide).

We pooled the plate counts for each replicate culture and calculated the MF according to the Poisson distribution (*34*). For a mutagenic response to be considered significant, the mean MF must be greater than the 95% confidence limit of the con-

current negative control (Dunnet's *t* test) and must be greater than the 99% upper confidence limit of the historical control observations (*35*). We estimated toxicity by cumulative growth of the cell cultures from the beginning of treatment to plating. Relative survival was calculated by dividing the cumulative growth of the sample-treated cultures by the cumulative growth of the negative control cultures.

Synthesis

(In this section, boldface numbers refer to the corresponding compounds in Figure 2.) CPP was prepared from pyrene according to the method published in the literature (*36*). AA and CPAA were both prepared from anthracene by methods we have described (*37,38*). For the synthesis of AP (Fig. 2), we treated phenanthrene with formaldehyde and hydrochloric acid (*36*). This gave a mixture of 1-(chloromethyl)phenanthrene (**1**) and 9-(chloromethyl)phenanthrene (**2**) (ratio 1:6). This mixture was treated under phase transfer catalytic conditions with sodium cyanide yielding a mixture of 1-(phenanthrene)acetonitrile (**3**) and 9-(phenanthrene)acetonitrile (**4**). Saponification with KOH in refluxing ethanol afforded the corresponding acetic acid derivatives (**5** and **6**). Conversion of the acids to their corresponding acid chlorides with oxalyl chloride (*37*) and subsequent treatment with $AlCl_3$ yielded a mixture of acephenanthren-4-one (**7**) and acephenanthren-5-one (**8**). $NaBH_4$ reduction of the ketones and subsequent dehydration with *p*-tol-uene sulfonic acid gave AP as yellow plates in 25% overall yield starting from phenanthrene.

For the preparation of CPAP (Fig. 2), we reduced the mixture of **7** and **8** under Wolff-Kishner conditions to yield acephenanthrene (**9**). Acephenanthrene was treated with oxalyl chloride with $AlCl_3$ as catalyst. In this way the second five-membered ring was introduced, forming 6,7-dihydrocyclopent[*hi*]acephenanthrene-4,5-dione (**10**). Wolff-Kishner reduction afforded 4,5,6,7-tetrahydrocyclopent[*hi*]acephenanthrylene (**11**). Treatment of compound **11** with 2,3-dicholoro-5,6-dicyano-1,4-benzoquinone (DDQ) in toluene led to the fully conjugated CPAP as dark-yellow plates.

All reagents were commercially available and were used without further purification. Solvents were distilled before use and dried if necessary. Petroleum ether with a boiling range of 60–80°C was used. Silica gel (230-400 mesh) was supplied by Merck. Melting points were determined on a Pleuger-Büchi melting point apparatus and are uncorrected; 300 MHz ^{1}H-NMR spectra were recorded on a Bruker WM-300 spectrometer with $CDCl_3$ as solvent, unless stated otherwise. We used tetramethylsilane (d = 0) as an internal standard. Chemical shifts (d) are given in ppm and coupling constants (*J*) in hertz. UV-visible spectra were determined on a Varian DMS 200 spectrophotometer. The mass spectra were determined on a Varian MAT 711 mass spectrometer (70 eV, source temperature 150°C, inlet temperature as reported).

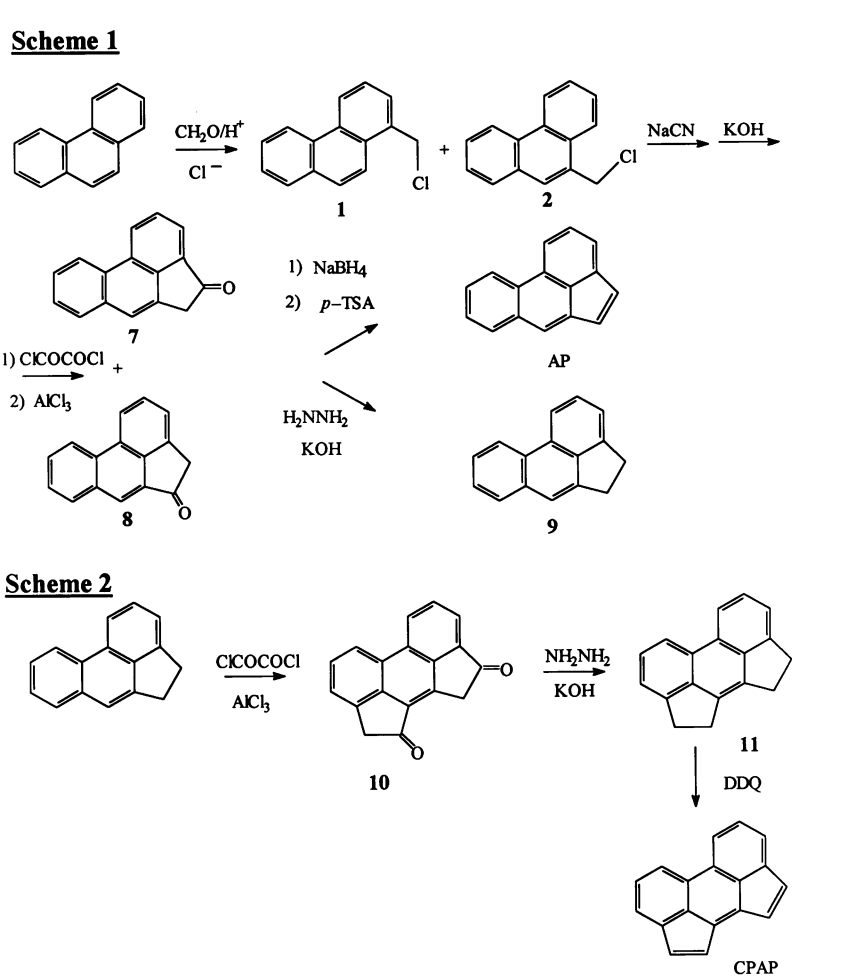


Figure 2. Synthesis of acephenanthrylene (scheme 1) and cyclopent[*hi*]acephenanthrylene (scheme 2). For a discussion of intermediates, see text.

1-(Chloromethyl)phenanthrene (1) and 9-(Chloromethyl)phenanthrene (2). Phenanthrene was chloromethylated according to the procedure of Fernàndez et al. (*39*). After work-up we purified the crude product by flash chromatography over a short column of silica. This procedure was sufficient to remove slow-running degradation components and to obtain the reaction products sufficiently pure for the next reaction step. ^{1}H-NMR revealed, next to 9-(chloromethyl)phenanthrene (**2**), some 1-(chloromethyl)phenanthrene (**1**) in the reaction mixture (ratio of **1** and **2**, 1:6). The total yield of compounds **1** and **2** was 95%. Compound **2**: ^{1}H-NMR: d 5.11 [s, 2H, -CH_2Cl]; 7.58–7.75 [m, 4H, H(2, 3,6,7)]; 7.82 [s, H(10)]; 7.88 [m, H(8)]; 8.22 [m, 2H, H(4,5)].

1-(Phenanthrene)acetonitrile (3) and 9-(Phenanthrene)acetonitrile (4). A mixture of **1** and **2** (21.7 g, 95 mmol), NaCN (7.5 g, 153 mmol) and triethylbenzylammonium chloride (3.2 g, 14.5 mmol) was suspended in a mixture of CH_2Cl_2 (30 ml) and H_2O (6 ml) under stirring. The suspension was refluxed for 16 hr and then allowed to cool to room temperature. We added a 5% NaOH solution and extracted the reaction mixture with CH_2Cl_2. We washed the organic layer twice with water and dried it over $MgSO_4$. An equal amount of petroleum ether was added, and the solution was filtered over a short column of silica and Hyflo to remove slow-running degradation products. Evaporation of the solvent yielded a mixture of compounds **3** and **4** (ratio 1:6; 18.7 g, 91%) as a light-brown oil. Compound **4**: ^{1}H-NMR: d 4.20 [s, 2H, CH_2CN] 7.61–7.77 [m, 5H, H(2,3,6,7,9)]; 7.89–7.93 [m, 2H, H(1,8)]; 8.67–8.79 [m, 2H, H(4,5)]. In the NMR spectrum the 1-isomer (**3**) is discernible by a singlet of the CH_2CN group at 4.27 ppm.

1-(Phenanthryl)acetic acid (5) and 9-(Phenanthryl)acetic acid (6). A mixture of compounds **3** and **4** (18.0 g, 83 mmol) was

dissolved in a mixture of ethanol (125 ml) and water (20 ml) under stirring. We added KOH (23.5 g) and refluxed the reaction mixture for 24 hr. The solution was allowed to cool to room temperature and water was added. We extracted the basic layer twice with diethyl ether to remove unreacted products and a small amount of residual phenanthrene. The basic layer was acidified with concentrated HCl and extracted twice with diethyl ether. The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated. Compounds **5** and **6** (ratio 1:6) were isolated as a white powder (14.7 g, 76%). Compound **6**: ^{1}H-NMR: d 4.17 [d, *J* 1.5, 2H H(a,a')]; 7.57-7.71 [m, 4H, H(2,3,6,7)]; 7.72 [s, H(10)]; 7.87 and 8.03 [2m, H(1 and 8)]; 8.67 and 8.75 [2m, H(4 and 5)]. MS (125°C) m/z (%): 236 (72), 222, (47), 205 (11), 191 (100), 189 (28), 177 (16), 176 (13), 165 (18). In the NMR spectrum the 1-isomer (**5**) is discernible by a singlet of the CH_2COOH group at 3.92 ppm.

Acephenanthren-4-one (7) and Acephenanthren-5-one (8). A mixture of crude compounds **5** and **6** (12.5 g, 53 mmol) was cyclized according to the procedure of Amin et al. (*40*). After work-up and column chromatography (silica; CH_2Cl_2/ petroleum ether 3:1), 5.0 g (44%) of a yellow mass was obtained, consisting of compounds **7** and **8** in a ratio of 4:1. Compound **7**: ^{1}H-NMR: d 3.72 [s, 2H, H(5,5')]; 7.56 [s, H(6)]; 7.63 [m, 2H, H(8,9)]; 7.77 [dd, *J* 8.0, 7.3 H(2)]; 7.87 [m, H(7)]; 7.98 [d, *J* 7.3 H(3)]; 8.53 [m, H(10)]; 8.63 [d, *J* 8.0, H(1)]. Compound **8**: ^{1}H-NMR: d 3.85 [s, 2H, H(4, 4')]; 7.56 [d, *J* 7.1, H(3)]; 7.67 [m, H(9)]; 7.72 [dd, *J* 8.4, 7.1, H(2)]; 7.79 [m, H(8)]; 8.09 [d, *J* 8.0, H(7)]; 8.21 [s, H(6)]; 8.42 [d, *J* 8.4, H((1)]; 8.65 [d, *J* 8.3, H(10)].

Acephenanthrylene. A mixture of compounds **7** and **8** (270 mg, 1.2 mmol) was dissolved in a mixture of CH_2Cl_2 (25 ml) and CH_3OH (25 ml). We added $NaBH_4$ (230 mg, 6 mmol) to the stirred solution. Stirring was continued for 30 min. Water was added, and the mixture was extracted with CH_2Cl_2. We dried the organic layer over $MgSO_4$ and evaporated it to dryness. The crude mixture of alcohols was dissolved in dry toluene (50 ml), and a catalytic amount of *p*-toluene sulfonic acid (15 mg) was added. The solution was refluxed for 30 min and allowed to cool to room temperature. We then washed the solution with a saturated $NaHCO_3$ solution and water and dried it over $MgSO_4$. The solvent was evaporated under reduced pressure. Column chromatography (silica; CH_2Cl_2/petroleum ether 1:9) and recrystallization (methanol) gave AP (215 mg, 86%) as yellow plates, melting point 140–141°C [literature values 140–141°C; (*40*) 141–142°C (*41*)]. AP: ^{1}H-NMR: d 7.10 [d, *J* 5.3, H(5)]; 7.20 [d, *J* 5.3 H(4)]; 7.60 [m H(8)]; 7.63-7.72 [m, 3H, H(2, 3,9)]; 7.99 [s, H(6)]; 8.00 [d, *J* 7.9, H(7)]; 8.39 [dd, *J* 7.6, 1.8, H(1)]; 8.64 [d, *J* 8.1, H (10)]. UV (cyclohexane) [λ_{max}, nm; (ε, l-mol^{-1}cm^{-1})]: 233 (27600), 264 (28800), 288 (9200), 300 (10400), 317 (6200), 330 (6750), 347 (6800), 365 (7900). Exact mass calculated for $C_{16}H_{10}$: 202.0782 m/z, found 202.0781 m/z, MS (25°C) m/z (%): 202 (100), 201 (13), 200 (17), 101 (19).

Acephenanthrene (9). A mixture of compounds **7** and **8** (5.0 g, 22.9 mmol) was suspended in diethylene glycol (150 ml) under an argon atmosphere. Hydrazine monohydrate (15 ml, 190 mmol) was added, and the reactants were dissolved under slight heating. After l hr we distilled off excess water and hydrazine until the temperature of the reaction mixture reached 160°C. We allowed the solution to cool to 80°C and added KOH (15 g) in portions. The solution was refluxed for 3 hr and finally cooled to room temperature. Water (150 ml) was added, and the reaction mixture was extracted with diethyl ether (2X). The combined organic layers were washed with water (2X) and dried over $MgSO_4$. Evaporation of the solvent gave the crude compound **9**. Purification by means of column chromatography (silica; CH_2Cl_2/petroleum ether 1:9) yielded acephenanthrene (4.05 g, 87%) as white plates, melting point 106–106.5°C [literature value 106°C (*42*)]. Compound **9**: ^{1}H-NMR: d 3.45 [s, 4H, H(4,4', 5,5')]; 7.46 [d, *J* 7.0, H(3)]; 7.52 [s, H(6)]; 7.57 [m, 2H, H(8,9)]; 7.62 [dd, *J* 8.1, 7.0, H(2)]; 7.85 [m, H(7)]; 8.31 [d, *J* 8.1, H(1)]; 8.59 [m, H(10)]. UV (cyclohexane), [λ_{max}, nm; (relative ε)]: 258 (1.00) 280 (0.18), 292 (0.17), 304 (0.22), 321 (0.011), 336 (0.022), 347 (0.009), 353 (0.028). MS (25°C) m/z (%): 204 (100), 203 (54), 202 (41), 201 (15), 200 (9), 101 (26).

6,7-Dihydrocyclopent[hi]acephenanthrylene-4,5-dione (10). Acephenanthrene (3.3 g. 16.2 mmol) was dissolved in CS_2 (50 ml). We added aluminum chloride (5.3 g, 40.3 mmol) and oxalyl chloride (3.1 g, 24.4 mmol) at 0°C under an atmosphere of argon. Stirring was continued for 5 hr at 0°C and for 16 hr at room temperature. We poured the reaction mixture onto ice (100 g) and acidified the water layer with 3N HCl and extracted it three times with dichloromethane. The organic layer was washed with water, dried over $MgSO_4$, and the solvent was evaporated. The orange residue was purified by column chromatography (silica; CH_2Cl_2). Com-pound **10** was collected as a yellow powder (750 mg, 18%), melting point 250-251°C (dec). Compound **10**: ^{1}H-NMR: d 3.58 [t, 2H, *J* 5.6, H(7,7')]; 3.75 [t, 2H, *J* 5.6, H(6,6 ')]; 7.66 [d, *J* 7.2, H(8)]; 7.82 [dd, *J* 8.2, 7.2, H(2)]; 7.86 [dd, *J* 8.1, 7.2, H(9)]; 8.08 [d, *J* 7.2, H(3)]; 8.35 [d, *J* 8.1, H(10)]; 8.73 [d, *J* 8.2, H(1)]. UV (meth-anol) [λ_{max}, nm; (relative ε)]: 252 (1.00), 280sh (0.70), 342sh (0.17), 380sh (0.07), MS (150°C): 258 (55), 230 (100), 202 (74), 201 (14), 200 (19), 113 (47).

4,5,6,7-Tetrahydrocyclopent[hi]acephenanthrylene (11). Compound **10** (750 mg, 2.9 mmol) was reduced with hydra-zine monohydrate (3.0 ml, 60 mmol) and KOH (3.0 g) in diethylene glycol (80 ml) in the same manner as described for the preparation of compound **9**. Column chromatography (silica; petroleum ether) and recrystallization (cyclohexane) furnished compound **11** (430 mg. 64%) as white plates, melting point 194–195°C. Compound **11**: ^{1}H-NMR: d 3.29 [m, 4H, H(4,4',7,7')]; 3.47 [m, 4H, H(5, 5',6,6')]; 7.43 [d, 2H, *J* 7.1, H(3,8)]; 7.53 [dd, 2H, *J* 8.0, 7.1, H(2,9)]; 8.23 [d, 2H, *J* 8.0, H(1,10)]. UV (cyclohexane) [λ_{max}, nm; (relative ε)]: 254 (0.74), 262 (1.00), 284 (0.15), 296 (0.17), 309 (0.27), 327 (0.015), 343 (0.032), 361 (0.052). Exact mass calculated for $C_{18}H_{14}$: 230.1096 m/z found: 230.1093 m/z. MS (75°C): 230 (100), 229 (50), 228 (21), 227 (20), 226 (19) 202 (10), 113 (18).

Cyclopent[hi]acephenanthrylene. Compound **11** (116 mg, 0.50 mmol) was dissolved in dry, freshly distilled toluene (40 ml) under an argon atmosphere, and DDQ (273 mg, 1.15 mmol) was added under stirring. We heated the solution to 80°C and kept it at that temperature for 1 hr. The solution was allowed to cool to room temperature and was filtered over a short column of silica. Evaporation of the solvent yielded CPAP as a light-orange solid. Column chromatography (silica; CH_2Cl_2/ petroleum ether 1:9) and recrystallization (cyclohexane) furnished CPAP as yellow needles, melting point 162– 163°C (dec). CPAP: ^{1}H-NMR: d 7.29 [d, 2H, *J* 5.3, H(5,6)]; 7.41 [d, 2H, *J* 5.3, H(4,7)]; 7.69 [dd, 2H *J* 7.6, 7.0 H(2,9)]; 7.73 [d, 2H, *J* 7.0 H(3,8)]; 8.38 [d, 2H *J* 7.6 H(1,10)]. ^{13}C NMR ($CDCl_3$): d 122.0, 123.0, 126.0, 126.1q, 128.0, 130.7q, 135.5, 136.1q, 139.6q ppm. UV (cyclohexane) [λ_{max}, nm; (ε, l-mol^{-1}cm^{-1})]: 224 (27300), 266sh (28500), 273 (34200), 326sh (11300), 338 (16600), 355 (20400), 374sh (6000), 393 (3800). Exact mass calculated for $C_{18}H_{10}$: 226.0893 m/z found: 226.0782 m/z. MS (25°C): 226 (100), 225 (12), 224 (15), 113 (31), 112 (18).

Results and Discussion

Bacterial Mutagenicity Assay

The four $C_{18}H_{10}$ isomers were tested in a forward mutation assay based on *S. typhimurium* as described in Methods. Additional details concerning this assay have

been published elsewhere (*28,29*). All isomers were tested in the presence of an exogenous metabolizing enzyme system (PMS). Such enzyme mixtures are generally required to obtain a mutagenic result for PAHs with bacterial assays.

The results for two $C_{18}H_{10}$ isomers are shown in Figure 3a and b, and these can be compared with the result for benzo[*a*]-pyrene (BaP) shown in Figure 3c. All compounds were tested with identical batches of bacteria and PMS to facilitate comparison. The Y-scale range for MF ($0–80 \times 10^{-5}$) is the same for the three in Figure 2. Error bars reflect the 99% confidence intervals for each determination.

As evidenced from this Figure 3, both BF and CPP are roughly twice as mutagenic as BaP. In all cases, toxicity is very low at the dose levels tested, as indicated by survival values near 100%. Mutagenicity results for CPAP and CPAA are shown in Figure 4. As illustrated in Figure 4a, CPAA is the most potent bacterial mutagen of the four $C_{18}H_{10}$ isomers tested and is approximately three times as mutagenic as BaP in this assay. CPAP, although yielding statistically significant mutagenicity results, is relatively inactive compared with BaP.

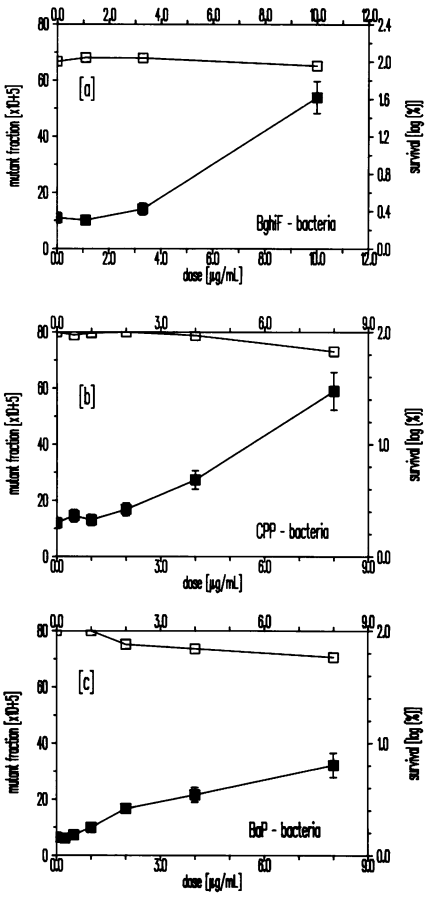


Figure 3. Bacterial mutagenicity results for two $C_{18}H_{10}$ PAH isomers and a benzo[*a*]pyrene reference sample. Dose–response curves were obtained from duplicate forward mutation assays with *S. typhimurium* in the presence of an exogenous metabolizing enzyme system. Mutagenicity data (lower curves) are represented by filled squares and are indexed to the left vertical scale; the corresponding toxicity data (upper curves) are represented by open squares and are indexed to the right vertical scale. Error bars for the mutagenicity data represent SDs. (a) Benzo-[*ghi*]fluoranthene; (b) cyclopenta[*cd*]pyrene; (c) benzo[*a*]pyrene.

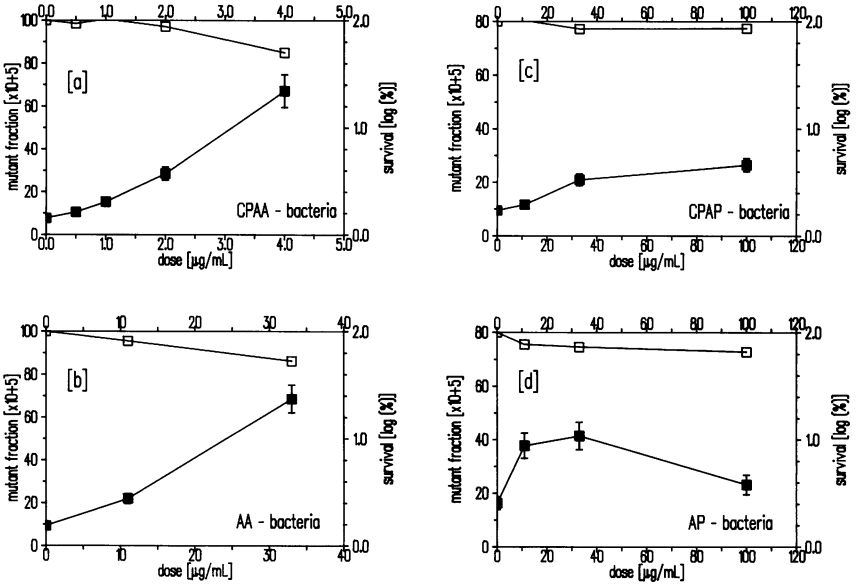


Figure 4. Bacterial mutagenicity results for two dicyclopenta-fused $C_{18}H_{10}$ PAH isomers and their monocyclopenta-fused analogs. Dose–response curves were obtained from duplicate forward mutation assays with *S. typhimurium* in the presence of an exogenous metabolizing enzyme system. Mutagenicity data (lower curves) are represented by filled squares and are indexed to the left vertical scale; the corresponding toxicity data (upper curves) are represented by open squares and are indexed to the right vertical scale. Error bars for the mutagenicity data represent SDs. (a) Cyclopent[*hi*]aceanthrylene; (b) aceanthrylene; (c) cyclopent[*hi*]acephenanthrylene; (d) acephenanthrylene.

Bacterial mutagenicity results for CPAA and CPAP were compared with those of their monocyclopenta-fused analogs, AA and AP. In Figure 4a and b, results for CPAA are compared with those for AA; in Figure 4c and 4d, results for CPAP are compared with those for AP. Although results for CPAP and AP were similar, the mutagenic activity of CPAA was an order of magnitude greater than that of AA. When comparing results for CPAA and AA, it should be noted that horizontal scale (dose) values are roughly an order of magnitude lower for CPAA (0–5 µg/mL) than for AA (0–40 µg/ml). CPAA is one of the most potent bacterial mutagens tested to date in our forward mutation assay.

Human Cell Mutagenicity Assay

In addition to the bacterial assay, the compounds were also tested for mutagenic activity at the *tk* locus in MCL-3 cells. Results for BF, CPP, and BaP are shown in Figure 5a, b and c, respectively. For this assay, error bars reflect 1 SD from the mean value. Mutagenicity values on the vertical axes are in units of 1×10^{-6} mutant fraction or parts per million. As shown in Figure 5, BaP is a very potent mutagen in this assay, showing statistically significant mutagenic activity below 0.5 µg/ml. CPP is also an important human cell mutagen, but it is less active than BaP in this assay.

In contrast, BF (Fig. 5a) is inactive in this human cell assay but quite active in bacteria as shown earlier (Fig. 3a). It should be noted that the BF analogs fluoranthene, benzo[*b*]fluoranthene, benzo-[*j*]fluoranthene, and benzo[*k*]fluoranthene (each has an internal cyclopenta-fused ring) are also inactive in the MCL-3 assay. Fluoranthene, however, is active in our bacterial assay (*27*). Presumably, the MCL-3 line of human cells lacks the enzyme(s) necessary for metabolic activation of fluoranthene-based PAHs.

Human cell mutagenicity results for CPAA and CPAP were compared with those of their monocyclopenta-fused analogs AA and AP. Results for CPAA compared with those of AA are shown in Figure 6a and b, respectively, and results for CPAP compared with those of AP are shown in Figure 6c and d, respectively. Comparing the results for CPAP and AP, it is seen that both PAHs are negative in this assay. On the other hand, both CPAA and AA are mutagenic. When comparing results for CPAA and AA, it should be noted that the horizontal scale (dose) is a factor of 10 smaller for CPAA

(0.0–0.9 μg/ml) than for AA (0–9 μg/ml). Comparing the data in Figure 6a and b, it is seen that the human cell mutagenicity of CPAA is more than an order of magnitude greater than that of AA.

Considering both the bacterial and human cell data, it can be hypothesized that the addition of peripherally fused cyclopenta groups to anthracene-based molecules will have a significant biological effect, but the addition of the same groups to phenanthrene-based structures will have little effect. No adequate explanation of the differences in the mutagenicity of these cyclopenta-fused PAHs can be offered until more information becomes available on the metabolism of these unique compounds.

Chemical Identification

Identification of $C_{18}H_{10}$ PAH isomers in complex mixtures is hampered by a number of technical difficulties; however, the lack of reference standards has been the major obstacle. In this study, the availability of four $C_{18}H_{10}$ isomers has enabled us to identify unequivocally the major $C_{18}H_{10}$ species emitted from a range of combustors and also has allowed us to shed some light on the effectiveness of selected analytical techniques for the analysis of these species in complex mixtures.

GC/MS is currently the method of choice for the separation and identification of PAHs (*24*); however, even GC/MS suffers from some important drawbacks when used for the analysis of complex mixtures for $C_{18}H_{10}$ PAHs. For example, the similarity of mass spectra of PAHs having the same molecular composition prevents the differentiation of isomers, while at the same time, it is often difficult to completely resolve $C_{18}H_{10}$ isomers from interfering species. In addition, coeluting $C_{18}H_{12}$ PAHs can mask contributions from less abundant $C_{18}H_{10}$ PAHs because the former give m/z 226 fragment ions, which can be mistaken for $C_{18}H_{10}$ molecular ions.

The introduction of diode array spectral data acquisition in HPLC (HPLC/DAD) has provided the potential to differentiate PAH isomers because PAHs generally give highly characteristic UV spectra, even for isomers. However, even with HPLC/DAD, the analysis of complex mixtures for $C_{18}H_{10}$ PAHs was found to give some difficulties: Even under currently optimal HPLC conditions, CPP and BF generally coelute, and unfortunately, have similar UV spectra. Moreover, it was found that the UV spectra of CPP, BF, CPAA, and CPAP could be masked beyond recognition by contributions from other coeluting PAHs (e.g., $C_{18}H_{12}$ isomers and alkyl-$C_{16}H_{10}$ PAHs).

Through the combined use of HPLC/DAD and GC/MS and by the use of the $C_{18}H_{10}$ reference standards, unequivocal identification of CPP, CPAP, and BF was made for a number of combustion samples. Comprehensive chemical analysis results for a range of combustors and pyrolyzers will be presented elsewhere; however, our present work focuses on the identification of $C_{18}H_{10}$ isomers in combustion products from a jet-stirred/plug-flow reactor, a research combustor that gives results typical of a number of practical combustion systems.

The jet-stirred/plug-flow reactor was designed to provide well-defined combustion conditions, and its use is part of an ongoing program aimed at understanding and controlling the combustion chemistry responsible for mutagen formation. Detailed descriptions of this combustor are available elsewhere (*23–25*).

Figure 7 shows a portion of an HPLC/DAD total-absorbance chromatogram obtained for an ethylene combustion sample from the combustor. Conditions are given in the Methods. The wide-band absorbance (236–500 nm) in absorbance units is plotted against the elution volume in milliliters. For PAHs, the wide-band absorbance is roughly proportional to mass (*27*). In Figure 7, the major peak, centered at 25 ml, was identified as CPP from the

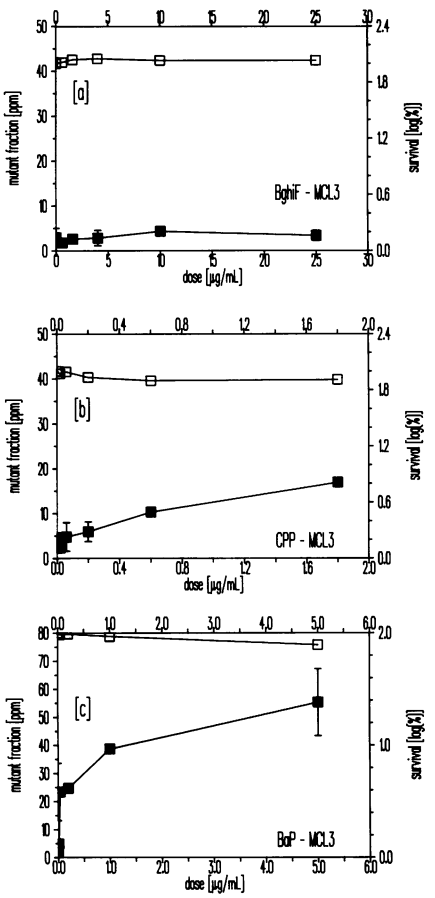


Figure 5. Human cell mutagenicity results for $C_{18}H_{10}$ polycyclic aromatic hydrocarbons and a benzo[*a*]pyrene reference sample. Mutagenicity data (lower curves) from duplicate experiments are represented by filled squares and are indexed to the left vertical scale; the corresponding toxicity data (upper curves) are represented by open squares and are indexed to the right vertical scale. Error bars for the mutagenicity data represent SDs. (a) Benzo[*ghi*]fluoranthene; (b) cyclopenta[*cd*]pyrene; (c) benzo[*a*]pyrene.

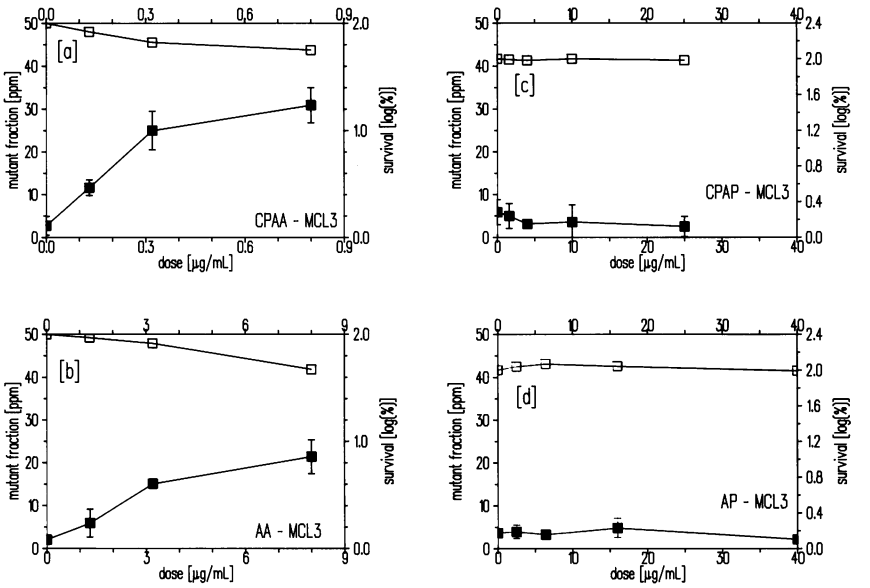


Figure 6. Human cell mutagenicity results for two dicyclopenta-fused $C_{18}H_{10}$ PAH isomers and their monocyclopenta-fused analogs. Mutagenicity data (lower curves) are represented by filled squares and are indexed to the left vertical scale; the corresponding toxicity data (upper curves) are represented by open squares and are indexed to the right vertical scale. Error bars for the mutagenicity data represent the SDs. (a) Cyclopent[*hi*]aceanthrylene; (b) aceanthrylene; (c) cyclopent[*hi*]acephenanthrylene; (d) acephenanthrylene.

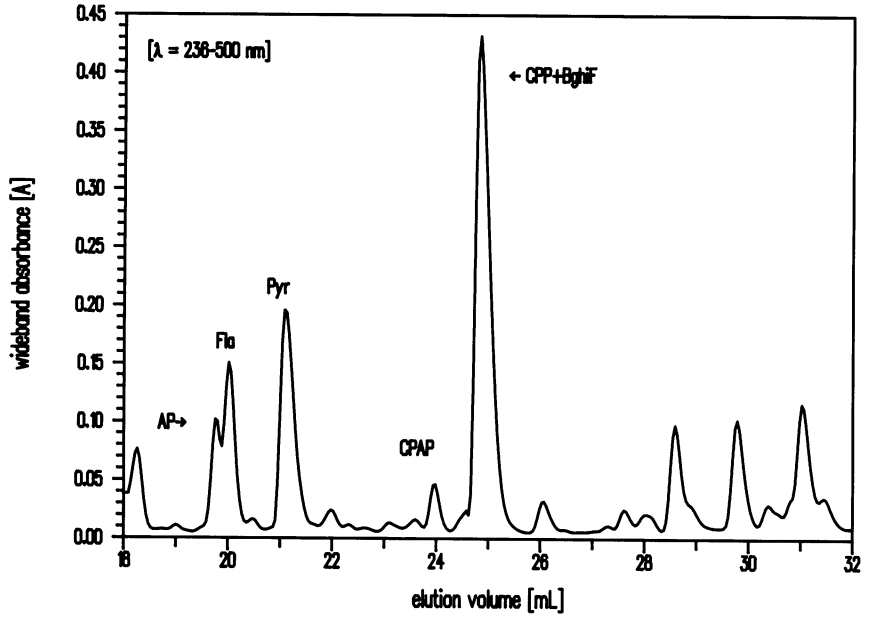


Figure 7. Total absorbance chromatograms obtained over the 236–500 nm wavelength interval showing the presence of $C_{18}H_{10}$ isomers cyclopenta[*cd*]pyrene and cyclopent[*hi*]acephenanthrylene in a sample produced from the combustion of ethylene in a jet-stirred/plug-flow reactor. See Methods for details. Other PAHs identified in the samples include acephenanthrylene, fluoranthene, and pyrene.

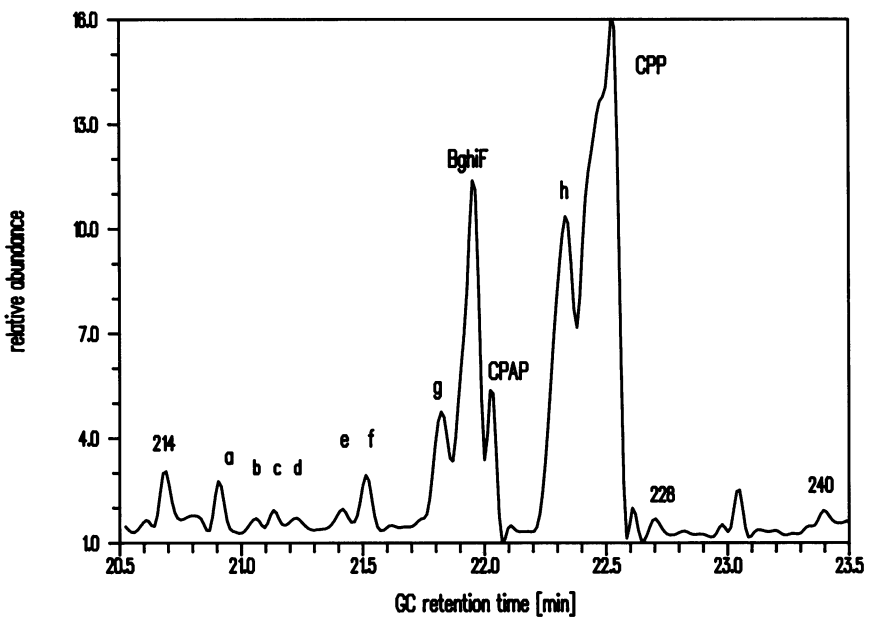


Figure 8. Mass chromatogram of m/e 226 ($C_{18}H_{10}$) for an ethylene combustion sample taken from a jet-stirred/plug-flow combustor. Three $C_{18}H_{10}$ isomers are identified: cyclopenta[*cd*]pyrene, benzo[*ghi*]fluoranthene, and cyclopent[*hi*]acephenanthrylene. A number of other $C_{18}H_{10}$ isomers, identified through their mass spectra, are indicated by peaks *a* – *h*.

HPLC/DAD data; however, further analysis by GC/MS showed that the major peak also contained BF. Quantitative analysis by GC/flame ionization detection gave a value of 16% for BF. The other $C_{18}H_{10}$ PAHs identified in this sample was CPAP, which was completely resolved from the CPP + BF peak. The earlier-eluting peaks annotated in Figure 7 are those of AP, fluoranthene, and pyrene.

Because our combustion conditions do not promote the formation of $C_{18}H_{12}$ PAHs (e.g., chrysene, benz[*a*]anthracene) or other interfering species, the HPLC chromatogram appears quite simple. However, when an HPLC fraction containing $C_{18}H_{10}$ PAH isomers was collected and analyzed by GC/MS, the additional resolution provided by a capillary GC column revealed a more complex picture.

Figure 8 shows the $C_{18}H_{10}$ mass chromatogram (m/e 226) for another ethylene combustion sample taken from our jet-stirred/plug flow combustor. The three $C_{18}H_{10}$ isomers (CPP, BF, CPAP) that were incompletely resolved by HPLC (Fig. 7) are better resolved by capillary gas chromatography. Moreover, at increased resolution, the presence of additional isomers is revealed. A number of other $C_{18}H_{10}$ isomers, identified through their mass spectra, were also found in the sample. They are indicated by peaks *a*–*h* in Figure 8. One of these additional isomers is likely to be cyclopenta[*cd*]fluoranthene. Other $C_{18}H_{10}$ PAHs can be formed in the combustor through the addition of an acetylene group to a $C_{16}H_{10}$ PAH (e.g., fluoranthene, pyrene). Evidence for the possible existence of these types of structures is provided by the presence in the sample of phenyl acetylene, 2-ethynyl naphthalene, and a number of ethynyl acenaphthylenes (*4*,*5*).

Also seen in Figure 8 are some other components eluting close to the $C_{18}H_{10}$ PAHs, and they are labeled with their molecular ion values obtained from their mass spectra. The small peak labeled 228 is a $C_{18}H_{12}$ isomer; the one labeled 240 gave a mass spectrum consistent with a $C_{18}H_{12}$ species incorporating an added methylene bridge. The 214 peak is thought to be a methylene-bridged acephenanthrylene.

Summary

In a forward mutation assay using on *S. typhimurium*, CPP, BF, and CPAA were roughly twice as potent as BaP, whereas CPAP was only slightly active. In a human cell mutagenicity assay using MCL-3 cells, a derivative of AHH-1 $TK^{+/-}$ cells containing higher native P450 1A1 activity, CPP and CPAA were strongly mutagenic but less active than BaP, while CPAP and BF were inactive at the dose levels tested. Bacterial and human cell mutagenicity results for CPAA and CPAP were compared with those of their monocyclopenta-fused analogs AA and acephenanthrylene AP. Mutagenicity results for both human cells and bacteria showed that the mutagenic activity of CPAP generally approximated that of AP: Both were inactive in human cells and weakly mutagenic in bacteria. In contrast, the mutagenic activity of CPAA was roughly an order of magnitude greater than that of AA in both assays. Results of chemical analyses showed that $C_{18}H_{10}$ CP-PAHs were abundant in every combustion and pyrolysis sample tested. Generally, CPP was the most abundant and the most frequently occurring $C_{18}H_{10}$ isomer, whereas CPAP was equally common but present but at lower levels. BF was also found in a number of samples, whereas CPAA was undetected.

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